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IDENTIFICATION OF A FEMALE-PRODUCED SEX PHEROMONE OF THE WESTERN CORN ROOTWORM

P.L. GUSS,[1] J.H. TUMLINSON,[2] P.E. SONNET,[2] and A.T. PROVEAUX[2]

[1]*Northern Grain Insects Research Laboratory, Agricultural Research Service, USDA, Brookings, South Dakota 57006.*
[2]*Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, USDA, Gainesville, Florida 32604.*

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Abstract—A sex pheromone has been isolated and identified from virgin females of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte. The synthesized compound, racemic 8-methyl-2-decanol propanoate, was equal in attraction to the natural pheromone when tested in the field as a trap bait against three taxa of *Diabrotica* known to respond to pheromone extracts from female WCR. Five taxa (*D. virgifera virgifera*; *D. virgifera zeae* Krysan and Smith, Mexican corn rootworm; *D. longicornis barberi* Smith and Lawrence, northern corn rootworm; *D. longicornis longicornis* (Say); and *D. porracea* Harold) were attracted to traps baited with 8-methyl-2-decanol propanoate. The response of male northern corn rootworms (NCR) in the field peaked at a relatively low concentration of 8-methyl-2-decanol propanoate and then was severely reduced at the higher concentrations tested. Conversely, the response of male WCR in the field continued to increase up to the highest dose tested.

Key Words—Chrysomelidae, *Diabrotica*, western corn rootworm, northern corn rootworm, Mexican corn rootworm, sex pheromone.

INTRODUCTION

The presence of a potent pheromone system in virgin females of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, has been known for some time (Guss, 1976; Bartelt and Chiang, 1977). Eariler studies by Ball and Chaudhury (1973) suggested the existence of a pheromone in the WCR of very low potency. These authors used extracts from field-collected insects of unknown mating history, and it was shown that mated WCR females do not attract males under field trapping conditions (Bartelt and Chiang, 1977).

Pheromone extracts from female WCR have been shown to attract males of the northern corn rootworm (NCR), *Diabrotica longicornis barberi* Smith and Lawrence, and live virgin females of both species appear equally attractive to males of either species when placed in field traps. A temporal difference in response between males of the two species was noted (Guss, 1976; Bartelt and Chiang, 1977). A recently described subspecies of *D. virgifera*, the Mexican corn rootworm (MCR), *Diabrotica virgifera zeae* Krysan and Smith, also readily responds to pheromone extracts from the WCR (Krysan et al., 1980). In this case, the temporal response is virtually identical to that of the male WCR; it was shown that these two subspecies interbreed in nature.

This paper reports the isolation, identification, and synthesis of a sex pheromone from WCR females and observations on the response of wild males of several *Diabrotica* taxa to the synthetic attractant. This is the first identification of a sex pheromone from a representative of the family Chrysomelidae, although a number of taxa in this family are significant economic pests throughout North America and much of the world.

METHODS AND MATERIALS

Pheromone Collection and Bioassay. Insects used in this study came from a laboratory colony reared according to Branson et al. (1975). Virgin females were isolated from males within 24 hr of emergence and held in screened cages (30 × 30 × 30 cm) for 3 days before being placed in pheromone collection chambers.

Initially, pheromone was absorbed on filter paper that had been in contact with virgin female beetles. Filter papers were shredded and extracted with 25% ether in hexane to yield a crude pheromone solution (Guss, 1976). This solution was subjected to thin-layer chromatography (TLC) on silica gel G with 5% ether in hexane as the solvent. The area of the chromatogram between R_f 0.23 and 0.37 was the only area containing pheromone activity. This area was scraped from the plate and extracted with 50% hexane–ether, and the resultant solution was suitable for gas–liquid chromatography (GLC) after volume reduction by evaporation at atmospheric pressure.

A new method was devised to collect larger quantities of pheromone. Approximately 1500–2000 virgin females were confined in a 6.5-liter glass cylindrical chamber. Air was filtered through charcoal, passed over the insects at 2.5 liter/min, and then through a filter (5.0 cm ID × 8.0 cm) containing ca. 5 g of Porapak Q® for pheromone absorption (Byrne et al., 1975). During pheromone collection, which was continuous, the insects were provided water and a dry diet mixed with a small amount of honey (Guss et al., 1976). Twice each week dead insects were removed and replaced with fresh females. Once each week all insects were removed from the chamber, the chamber was washed, and a fresh Porapak Q filter was installed.

Laboratory bioassay and field trapping experiments were conducted essentially as described earlier (Guss, 1976).

For laboratory bioassay, 4 or 5 male WCR beetles were placed in a disposable Petri dish (150 × 15 mm) and allowed to become acclimated for 15 min. Then a test compound in 1–5 μl of hexane was applied to a filter paper chip (5 mm^2), and after solvent evaporation (about 10 min), the treated chip was placed in the Petri dish. Positive responses consisted of orientation of the beetles toward the chip, distinctive antennal waving, and copulatory behavior toward the other males.

For field studies involving comparison of equal amounts of natural or synthetic pheromone, traps consisting of inverted 360-ml plastic coated cups were placed in corn fields on top of wooden stakes such that the top of the trap was about first ear height or about 1 m from the ground. Pheromone in hexane containing 10% trioctanoin was dispensed from cylindrical cotton wicks (30 × 10 mm) attached to the top of the trap. The traps were coated with Stickem Special®.

For field studies involving several concentrations of synthetic pheromone, the pheromone was dispensed from red rubber septa. In this case, no trioctanoin was added to the pheromone solution.

Pheromone Purification. Crude pheromone was extracted from the Porapak Q by back-flushing with 10 ml of 25% ether in hexane or 25% ether in pentane. Subsequent Soxhlet extraction of the filter material with ether and GLC analysis of that extract indicated that only trace amounts of pheromone remained on the Porapak after the first extraction. The volume of the eluent was reduced by evaporation at atmospheric pressure, and the ether–hexane extract was subjected to micropreparative GLC without further purification.

Micropreparative GLC for isolation of the pheromone was performed on a Varian model 1400® gas chromatograph equipped with a flame ionization detector. Stainless-steel columns were packed with 30% DEGS on 60/80 mesh Chromosorb-W® (3.2 mm OD × 9.1 m) and 1.5% OV-101® on 100/120 mesh Chromosorb G-HP® (3.2 mm OD × 1.5 m). Inlet temperature was 185° C and 150° C, respectively; the detector temperature was 250° C. Column temperatures were: DEGS, 160°, and OV-101, 120° C isothermal. A glass column (2 mm ID × 2 m) packed with 6% Carbowax 20M® on 120/140 mesh Chromosorb W was installed in an identical gas chromatograph and operated at 160° C with an injector temperature of 210° C. Carrier gas (N_2) flow rate through all columns was 20 ml/min.

The chromatographs were modified to accommodate a 90:10 effluent splitter and external fraction collector (Brownlee and Silverstein, 1968). Dry ice and acetone was the coolant for the fraction collector. Fractions were collected in 1.5 × 305-mm capillary tubes and were subsequently eluted with a minimal volume of hexane.

Analytical GLC was performed on 0.25-mm (ID) glass capillary columns

(Heath et al., 1980). An SP2340® column, 66 m long, was placed in a Hewlett-Packard model 5710A® gas chromatograph equipped with a model 18740B split/splitless injector. This system was operated at a N_2 carrier gas flow of 9.5 cm/sec. The column temperature was maintained at 60°C for 2 min after injection and then programed at 32°/min to 120°C. The splitless delay was 60 sec. An OV-101 column, 31 m long, in a Varian model 1200® GC equipped with a split injection system was operated at a He carrier gas flow of 18 cm/sec, a column temperature of 150°C, and an inlet split ratio of 100:1. A cholesteryl cinnamate column, 20 m long, was operated at the same conditions in a Varian model 1400 GC equipped with a split injection system.

Preparative liquid chromatography was performed on a 1.27 × 25-cm stainless-steel column packed with 5 μm Licrosorb® SI60 (Heath et al., 1978). A Lab Data Control Constametric II G Pump® delivered the hexane–ether (99:1) mobile phase at 3 ml/min, and the eluting components were detected with a Waters model R401® differential refractometer.

Pheromone Identification. Alkaline hydrolysis, hydrogenation, and bromination were conducted on small amounts of pheromone samples partially purified by TLC. Alkaline hydrolysis was carried out in 2 ml of 2 N KOH in 50% ethanol. The mixture was refluxed for 2 hr in a boiling water bath. After cooling, 1 ml H_2O was added, and the mixture was extracted with 3 ml hexane. The extraction procedure was repeated after acidification of the reaction mixture with 0.4 ml 10 N H_2SO_4. Both extracts were bioassayed in the laboratory and field.

Catalytic hydrogenation was carried out in a laboratory constructed microhydrogenator (Farquhar et al., 1959) in methanol with platinum oxide as the catalyst. The mixture was agitated vigorously under an atmosphere of hydrogen for 2 hr at ambient temperature. After removal of the catalyst, the mixture was bioassayed in the laboratory and field.

Bromination was conducted in methylene chloride containing an excess of Br_2. The mixture was allowed to react overnight at ambient temperature. Following this, 1 ml of 1% sodium bisulfite was added and the mixture shaken to convert excess Br_2 to HBr. The organic layer was washed four times with 2 ml H_2O before being used for laboratory and field bioassays.

Mass spectral data were obtained with a Finnegan model 3200® mass spectrometer equipped with both chemical ionization and electron impact sources. A Varian model 1400 gas chromatograph equipped with a 5% OV-1 column, 2.2 mm (ID) × 2 m, served to introduce samples to the CI source. Methane was employed as reagent gas and GLC carrier gas. The EI source was served by a Finnegan model 9500® gas chromatograph equipped with an OV-1 column of the same dimensions. Helium was normally used as the carrier gas.

Hydrogenolysis was carried out in the injector of the gas chromatograph that served the EI source by the method of Beroza and Sarmiento (1963, 1964). About 6 cm of a 3.2-mm (ID) stainless-steel tube was filled with neutral Pd catalyst and placed in the injector port ahead of the OV-1 column. The catalyst was maintained at 225°C for the hydrogenolysis, and H_2 was used as the carrier gas at 30 ml/min.

RESULTS AND DISCUSSION

Early isolation studies were conducted with pheromone collected by the filter paper method. Difficulties encountered in rearing large numbers of insects coupled with the fact that the WCR apparently produces only small amounts of pheromone resulted in the collection of only nanogram quantities. Nevertheless, because the WCR male responds to nanogram quantities of the pheromone, both in the laboratory and in the field, it was possible to determine separation parameters by chromatographic fractionation coupled with laboratory and especially field bioassay.

The laboratory bioassay (Guss, 1976) was shown to be definitive for the presence of the pheromone when used in conjunction with TLC, i.e., a positive response with the laboratory bioassay always correlated with a positive response in the field. Erratic results, however, were obtained with the laboratory bioassay when testing GLC fractions, necessitating heavy reliance on field-trapping experiments to identify GLC fractions containing pheromone. Attempts to demonstrate pheromone activity in a greenhouse or large-chamber situation with laboratory-reared males failed.

Partially purified pheromone obtained from TLC was first subjected to GLC on 30% DEGS. Fractions were collected every 4 min and checked for the presence of pheromone by field bioassay. Only that fraction eluting between 20 and 24 min was attractive to male WCR. Subdivision of this area of the chromatogram and subsequent bioassay showed a single peak of activity corresponding to a retention index of 18.40 (Kovats, 1965).

Chromatography on 1.5% OV-101 of partially purified pheromone from TLC as well as rechromatography of the active fraction from DEGS again produced a single active fraction eluting between 20 and 24 min. The active peak on this column had a retention index of 14.55.

Alkaline hydrolysis of the partially purified pheromone from TLC by 15% KOH in ethanol completely destroyed pheromone activity, but catalytic hydrogenation or bromination had no apparent effect. These results suggested that the pheromone was an ester containing no olefinic bonds.

Improved rearing techniques increased the number of virgin females available for pheromone production to the point that it became feasible to undertake the isolation of microgram quantities of pheromone. Thus, the Porapak Q method was developed, and approximately 200,000 beetles over a 2-year span yielded about 10 μg of pheromone. The material extracted from Porapak Q was purified by sequential chromatography on DEGS and OV-

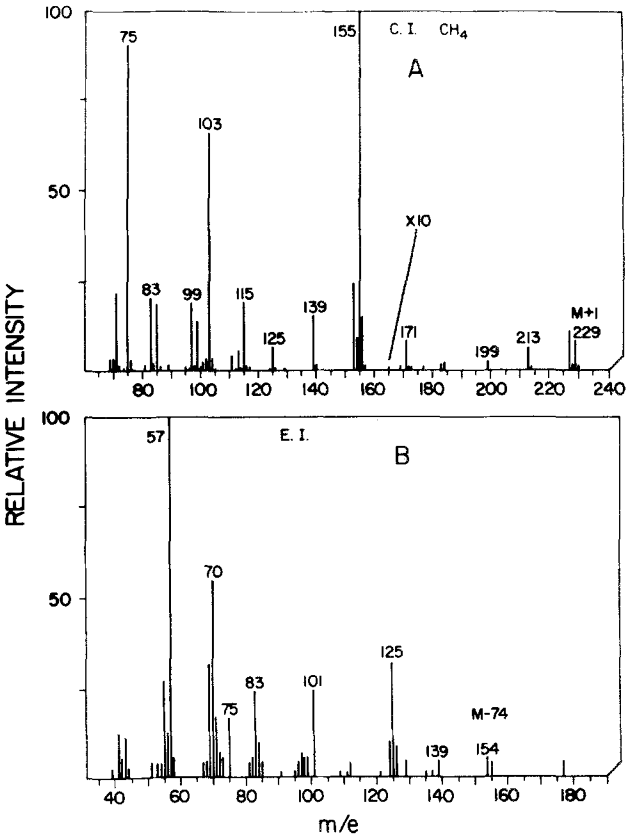

FIG. 1. (A) Chemical ionization (CH_4) mass spectrum of *D. virgifera* pheromone. (B) Electron impact mass spectrum of *D. virgifera* pheromone.

101 (see above) to yield a single fraction that was active in both laboratory and field bioassays. Male NCR were also attracted to those fractions attractive to the WCR. This was not unexpected since it had been shown earlier that extracts as well as live virgin females of either species attracted males of both species (Guss, 1976; Bartelt and Chiang, 1977).

Analysis on the OV-101 capillary column of the active material collected from the packed OV-101 column indicated that it contained about 5% of an impurity eluting immediately after the major peak. This impurity appeared as a shoulder on the major peak on the packed OV-101 column and could not be removed by chromatography on the other packed columns without sacrificing an unacceptable amount of the major peak which was known to be active.

The methane ionization mass spectrum of the major peak (Figure 1A) indicated this compound was a propionate ester of an 11-carbon saturated alcohol with diagnostic peaks at m/e 229 (M + 1), 227 (M − 1), 155 (M + 1 −

CH_3CH_2COOH), 75 ($CH_3CH_2COOH_2^+$), 103 ($CH_3CH_2COOHC_2H_5^+$), and 115 ($CH_3CH_2COOHC_3H_5^+$). The strong peaks, relative to M + 1, at 213 and 199 suggested the possibility of methyl substitution on the 3rd carbon from the hydrocarbon end of the alcohol moiety. The electron impact spectrum of this compound (Figure 1B) strengthened the evidence for a propionate with the peak at *m/e* 75, and the strong peak at *m/e* 101 indicated an alcohol function in the C-2 position of the chain. Jewett et al. (1976) suggested that a strong peak at *m/e* 101 is characteristic of a propionate function at the C-2 position and a branch at the C-3 position. However, the EI spectrum of authentic 2-undecanol propanoate, obtained under identical conditions, contained a peak at *m/e* 101 of about the same relative intensity as that found in the pheromone spectrum. The strong peak at 125 is characteristic of a methyl branch at either the C-3 or C-8 position of a 10-carbon chain, but its intensity suggested that a methyl at C-8 was more likely.

The mass spectrum of the product of hydrogenolysis of the pheromone in the gas chromatographic injector leading to the EI source is shown in Figure 2. The peaks at *m/e* 156 (M+) and 126 support the structure of 3-methyl decane for the hydrogenolysis product.

Thus all the mass spectral evidence supports the assignment of 8-methyl-2-decanol propanoate (I, Figure 3) for the structure of the pheromone, but 3-methyl-2-decanol propanoate could not be ruled out absolutely.

Synthesis of I. The synthesis of racemic 8-methyl-2-decanol propanoate, I (Figure 3) commenced with the reaction of methyl cyclopropyl ketone and ethyl magnesium bromide. The product was isomerized (Julia, 1961) producing the homoallylic bromide II (bp 76–82°C at 30 mm, rep. bp 76–80°C at 27

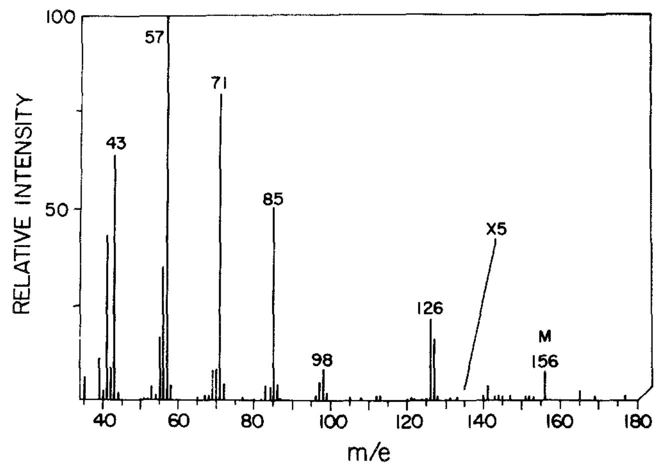

FIG. 2. Electron impact mass spectrum of hydrogenolysis product of *D. virgifera* pheromone.

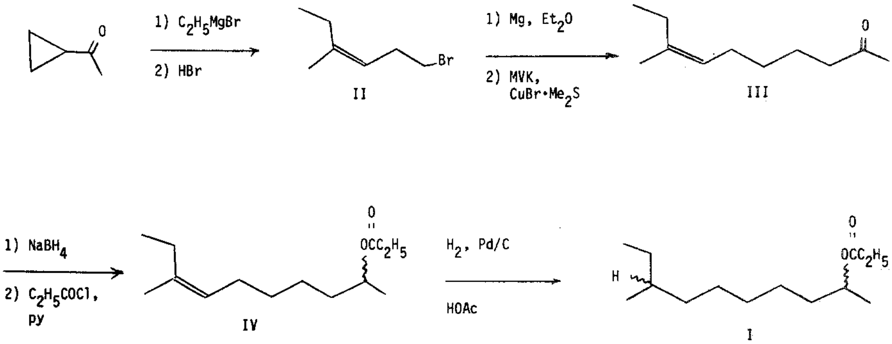

FIG. 3. Synthesis of racemic 8-methyl-2-decanol propanoate.

mm, yield: 74%). The bromide was converted into a Grignard reagent in ether and added to a stirred suspension of cuprous bromide–dimethyl sulfide (House et al., 1975) in an ethereal solution of freshly distilled methyl vinyl ketone. The ketonic product, III, was obtained pure after chromatography on silica gel separated it from the dimeric hydrocarbon formed from II; i.e., the Grignard preparation from II was beset by self-condensation. Although several alternative techniques and catalysts were examined, the study was not exhaustive. In addition, the procedure described provided a product free of the allylic alcohol that would (and generally did) derive from condensation of the Grignard reagent of II with the carbonyl group of methyl vinyl ketone. This inverse addition technique gave III in ca. 37% yield from II[IR(CCl_4) 1700 cm^{-1} (C=O)]. Ketone III was reduced with $NaBH_4$ in methanol to give the corresponding alcohol [IR (CCl_4) 3560 cm^{-1} (OH)] which was converted directly to the propionate ester IV using propionyl chloride in pyridine [bp 66°C (bath temp.) at 0.005 mm; IR (CCl_4) 1740 cm^{-1} (ester C=O); NMR (d_6-acetone) 5.0 (m, 1H, vinyl H), 2.28 (g, 2H, $J = 7$, $CH_3\underline{H}_2CO_2$), 1.18 (d, 3H, $J = 7$, $C\underline{H}_3CHO$), 1.07 ppm (t, 3H, $J = 7$, $C\underline{H}_3CH_2CO_2$); yield ca. 80–90% from ketone III). The saturated ester I was then obtained by hydrogenation over 5% Pd / C in acetic acid [bp 60–65° C (bath temp.) at 0.005 mm; IR (CCl_4) 1740 cm^{-1}; NMR (d_6-acetone) 4.86 (m, 1H, $CH_3C\underline{H}O$), 2.26 (g, 2H, $J = 7$, $CH_3C\underline{H}_2CO_2$), 1.17 (d, 3H, $J = 7$, $CH_3C\underline{H}O$), 1.06 ppm (t, 3H, $J = 7$, $CH_3C\underline{H}_2CO_2$)].

The synthesized racemic 8-methyl-2-decanol propanoate was purified by high-performance liquid chromatography (HPLC) on Licrosorb SI60 and subsequently by gas chromatography on Carbowax 20M. Analysis of the material collected from Carbowax 20M by capillary gas chromatography on OV-101, SP2340, and cholesteryl cinnamate indicated that it was greater than 99% pure and identical in retention time to the major peak of the active pheromone fraction collected from OV-101. Both the EI and CI spectra of 8-methyl-2-decanol propanoate were identical to the respective spectra of the major peak of the natural material.

TABLE 1. COMPARISON OF 8-METHYL-2-DECANOL PROPANOATE (8-MDP) AND NATURAL WCR PHEROMONE

Species	Average number of insects trapped		
	8-MDP	WCR pheromone	Unbaited control
D. virgifera zeae (MCR)	173[a]	180[a]	12
D. virgifera virgifera (WCR)	1703[b]	1868[b]	19
D. longicornis barberi (NCR)	1455[b]	1339[b]	145

[a]Average of 4 traps containing about 10 ng active compound. Data collected for 24-hr period near Beeville, Texas.
[b]Average of 2 traps containing about 350 ng active compound. Data collected for continuous 12-day period near Brookings, South Dakota.

Bioassay of I. Laboratory bioassay of synthesized racemic 8-methyl-2-decanol propanoate (I) elicited behavior from male WCR identical to that observed with the natural pheromone. Field assay of I was first carried out in late May 1980 in cornfields near Beeville, Texas. The corn rootworm population in these fields was exclusively Mexican corn rootworm (MCR), *D. v. zeae.* Traps containing as little as 10 ng of I were highly attractive to the MCR compared to unbaited control traps.

Data in Table 1 show the comparative attractive properties of 99+% pure I and unpurified natural WCR pheromone toward the MCR under field conditions. The concentraton of I and natural pheromone was ca. 10 ng as determined by GLC. Because of time limitations, the data in Table 1 concerning the MCR are from a single 24-hr period; nevertheless, the average number of beetles caught by each treatment strongly suggests that I is the active component in pheromone extracts from WCR females responsible for attracting MCR males.

Comparison of 99+% pure I and >90% pure natural pheromone in the presence of native populations WCR was carried out at Brookings, South Dakota, in early August of 1980. Data in Table 1 show the results of trapping for a continuous 12-day period. About 350 ng of I or natural pheromone were placed in each trap. The data show that I and the natural pheromone are equally attractive at these levels to both the WCR and the NCR. The identity of the impurity in the isolated WCR pheromone fraction was not determined. Since the natural and synthetic pheromone elicited identical responses when tested at the same concentration, this impurity apparently does not interfere with trap captures. The possibility that this compound plays some as yet undefined role in WCR chemical communication or that other active components of the WCR pheromone exist cannot be ruled out. Laboratory bioassays of GLC fractions of the natural extract indicated that other compounds may play a role, as yet undefined, in the communication of the

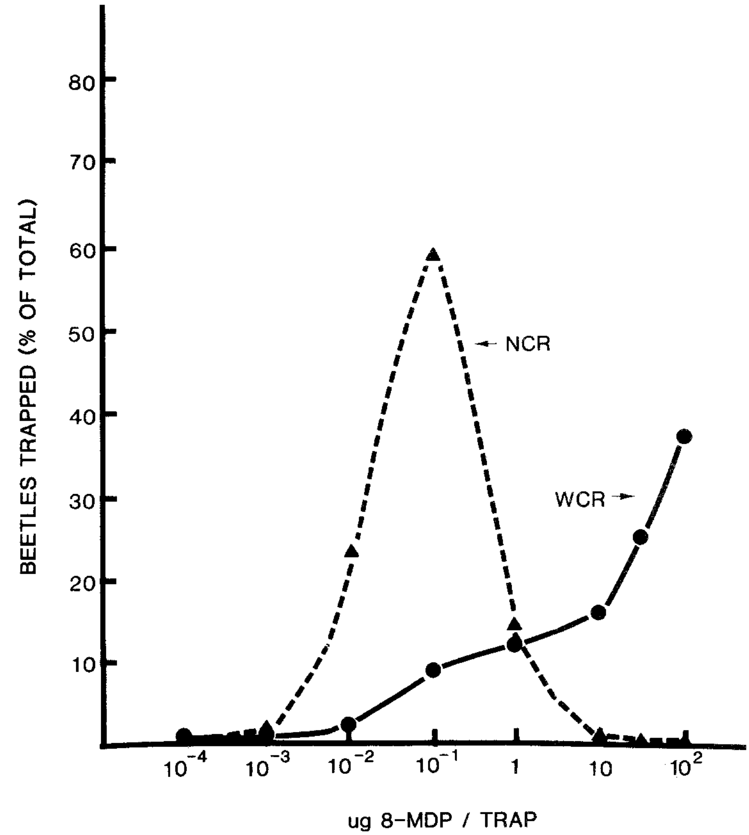

FIG. 4. Percentages of total WCR and NCR captured in field traps containing varying amounts of 8-methyl-2-decanol propanoate. Traps were located in a field containing a mixed population of WCR and NCR.

WCR. We therefore conclude that 8-methyl-2-decanol propanoate is identical to the major, if not the only, natural product produced by WCR females that is responsible for attraction of WCR males under field conditions.

Because only small amounts of natural pheromone were available for these studies, relatively small amounts of compounds were used in the comparative experiments. Another series of experiments was conducted in which a wide range of concentrations of I was placed in field traps in the presence of a mixed population of WCR and NCR. The results of this study, summarized in Figure 4, show that trap catches of WCR increased with increasing pheromone concentration while catches of the NCR peaked at a pheromone concentration of about 0.1 μg/trap and fell to zero at 10 μg/trap and above. The fact that the synthetic pheromone was racemic may have an influence on the results obtained. The effects of individual stereoisomers or blends thereof await the availability of these compounds in pure form.

In addition to the taxa mentioned above, at least two other diabroticites are known to be attracted to traps baited with I. Thus, in trapping experiments in Mexico (State of Jalisco) males of *D. porracea* Harold were found in small but significant numbers in I baited traps (T.F. Branson, personal communication). This species never appeared in unbaited control traps, and intensive personal searches failed to produce a single free moving specimen of either sex, indicating the scarcity of these insects in the trap area. Also, males of *D. longicornis longicornis* (Say), which closely resemble those of the northern corn rootworm, were attracted to I baited traps placed in wild cucurbit patches in Kansas (J.L. Krysan, personal communication).

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